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ORIGINAL ARTICLE

Reduction in circulating pro-angiogenic and pro-inflammatory factors is related to improved outcomes in patients with advanced pancreatic cancer treated with gemcitabine and intravenous omega-3 fish oil

Ali Arshad[1], Wen Y. Chung[1], William Steward[2], Matthew S. Metcalfe[1] & Ashley R. Dennison[1]

Departments of [1]Hepatobiliary and Pancreatic Surgery, and [2]Department of Medical Oncology, University Hospitals of Leicester, Leicester, UK

Abstract

Background: Pancreatic cancer is a rapidly progressive disease which is often only amenable to palliative treatment. Few patients respond to palliative chemotherapy, so surrogate markers indicating which patients are likely to respond to treatment are required. There is a well-established link between pro-inflammatory circulating cytokines and growth factors (CAF), and the development of neoplasia. Agents that may modulate these factors are of interest in developing potential novel therapeutic applications.

Methods: As part of a single-arm phase II trial in patients with advanced pancreatic cancer (APC) treated with gemcitabine and intravenous (i.v.) omega-3 rich lipid emulsion (n-3FA), serum samples were analysed for 14 CAF using a multiplex cytokine array. Baseline serum concentrations were correlated with overall (OS) and progression-free survival (PFS), and changes in concentration correlated with time and outcomes for CAF responders were analysed.

Results: Platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) concentrations reduced significantly with treatment over time. Low baseline interleukin (IL)-6 and -8 were correlated with improved OS. PDGF responders showed a tendency towards improved OS and FGF responders a significantly improved PFS.

Discussion: Treatment with gemcitabine plus i.v. n-3FA may reduce concentrations of CAF which may be associated with an improved outcome. Baseline IL-6 and -8 may be surrogate markers for outcome in patients with APC treated with this regimen.

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Correspondence

Ali Arshad, Department of Hepatobiliary and Pancreatic Surgery, Leicester General Hospital, Gwendolen Road, Leicester, LE5 4PW, UK. Tel: +44 116 258 8110. Fax: +44 116 258 4708. E-mail: ali.arshad@doctors.org.uk

Introduction

Pancreatic cancer is a rapidly progressive disease with a poor outcome: 80% of patients have surgically unresectable disease at presentation with a median survival of 6–12 months even with the best available palliative chemotherapy regimens.[1,2] Clearly novel agents which can target specific pathways in tumour progression are indicated together with biomarkers which can identify those patients who will respond to treatment. There is a clear association between angiogenesis and the development of most human solid tumours, evidenced by data showing increased serum concentrations of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), epidermal growth factor (EGF) and fibroblast growth factor (FGF) in these patients.[3–6] VEGF inhibition as an anti-angiogenic strategy for the

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treatment of solid tumours gained particular interest as a result of overexpression and its correlation with a poor outcome.[7,8] This was reinforced by an improved outcome compared with standard treatment in late phase randomized clinical trials using agents which target receptors for these factors, such as bevacizumab (VEGF-A: colorectal and lung), cetuximab (EGF: colorectal, head and neck cancer) and erlotinib (EGF: lung cancer). However, when applied to pancreatic cancer in randomized clinical trials, most of these strategies have proven to have no clinical benefit.[9,10] Only erlotinib was shown to have an overall survival (OS) benefit, and although statistically significant, this has not translated into widespread use as the clinical difference was marginal at best (10 days OS improvement over gemcitabine alone).[11] This may be because of the fact that pancreatic cancers are not highly vascular tumours, and that they usually have a dense stromal reaction around the tumour which may protect neoplastic cells from targeted agents. Studies examining changes in pro-angiogenic cytokines and growth factors (CAF) in advanced pancreatic cancer (APC) patients have shown significantly increased expression of PDGF, VEGF and EGF compared with healthy controls.[12] High concentrations of VEGF have been shown to be related to poor outcome in previous studies of patients with APC.[13–15] The role of PDGF in neoplasia is less clear. PDGF-BB stimulation may enhance invasiveness in pre-clinical cell line models.[16] There may be a synergistic role for PDGF and VEGF in tumourigenesis, with PDGF blockade potentiating the anti-neoplastic action of VEGF blockade in cell lines.[17] PDGF expression is correlated with a poor clinical outcome in gastric cancer and osteosarcoma patients.[18,19]

In pre-clinical experiments and clinical trials, omega-3 fatty acids (n-3FA) have been shown to be able to modulate CAFs and therefore have an anti-angiogenic potential.[20,21] They are rapidly incorporated into cell membrane phospholipid bilayers by competition with omega-6 fatty acids. Cyclo-oxygenase-2 acting on n-3FA produces metabolites which are far less pro-inflammatory and pro-angiogenic than their n-6FA related counterparts. These metabolites downregulate transcription of pro-angiogenic growth factors and n-3FA has been shown to reduce expression of PDGF both *in vivo* and in randomized clinical trials using healthy volunteers.[22]

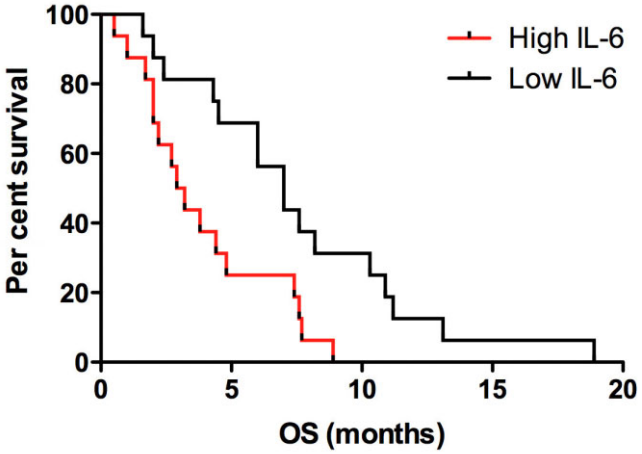
Methods

Patients with locally advanced or metastatic pancreatic cancer not suitable for surgical resection but eligible for gemcitabine chemotherapy were enrolled in a single-arm phase II clinical trial (clinicaltrials.gov registration NCT01019382). All patients were of Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1 and none had undergone any prior chemotherapy treatment for any reason. Patients underwent treatment with gemcitabine (1000 mg/m^3, Gemzar®; Eli Lilly Co., Indianapolis, IN, USA) immediately followed by intravenous (i.v.) n-3FA-rich lipid emulsion (up to 100 g, Lipidem®, B Braun, Melsungen, Germany) weekly for 3 weeks followed by a rest week. This was continued until tumour progression, subject death or withdrawal. As part of this trial, which had clinical primary outcome measures, baseline concentrations and changes in serum CAFs were evaluated and correlated with clinical outcome.

Whole blood was taken from the patients immediately prior to treatment each week to minimize transient changes associated with chemotherapy. This was transferred to a serum gel tube which was centrifuged within 30 min at 1500 g for 15 min at 4°C. The serum was transferred to Eppendorf tubes and stored at −80°C. At the time of analysis, the serum was thawed and subject to cytokine concentration quantification using a multiplex cytokine array (Aushon biosystems).

The following pro-inflammatory and pro-angiogenic cytokines were evaluated in the multiplex array: interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α), IL-6 and -8, interferon gamma (IFN-γ) VEGF-A, VEGF-C and VEGF-D, Tumour necrosis factor alpha-related apoptosis inducing ligand (TRAIL), Receptor activator of nuclear factor kappa beta ligand (RANKL), PDGF, hepatocyte growth factor (HGF), FGF and EGF.

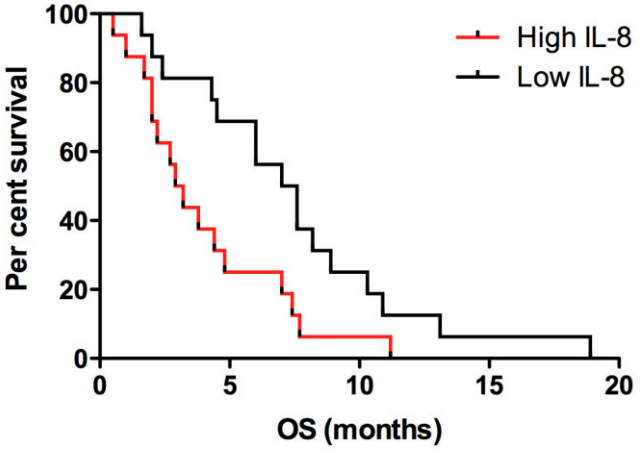
The serum was thawed on ice and pipetted into the plate in duplicate. The serum samples were run neat either in a 1:2 or a 1:4 dilution depending on the expected concentration of factors to be detected and the dynamic range of the array. The standards were made up in duplicate and the appropriate dilutions transferred to the plate. Once all the wells were filled with either standards (first 16 wells) or serum samples (next 80 wells), the plate was gently agitated for 1 h using an automated plate shaker. The plate was then thoroughly washed manually using Aushon custom wash, and biotinylated antibody added to each well. This was then agitated again for 30 min and washed manually three times. Streptavadin-horseradish peroxidase conjugate was then added to each well and the plate agitated for 30 min and washed three times. Finally a luminal-based substrate was added and the plate read within 2 min in the custom-built CCD camera image detector. Aushon Searchlight software was used to capture and analyse the image to provide a concentration in each well of each sample to be analysed compared with the standards. The concentrations were then entered into an Excel spreadsheet to provide data on changes with treatment and time for each patient. The changes over time in the logarithms of the concentrations of each factor were modelled using a random coefficients model fitted using xtmixed in STATA software. This model fits a linear regression in which both the intercept and the slope are allowed to vary randomly between individuals. In order to define CAF responders, those patients who had a > 30% decrease in CAF concentration during the treatment were defined as responders for that particular CAF. Patients were divided into either high or low expressors of CAFs at baseline around the median. Kaplan–Meir survival curves were constructed using Graphpad software to analyse OS and progression-free survival (PFS) relationships with baseline cytokine concentrations and cytokine responders.



Numbers at risk

OS (months)	0	2	4	6	8	10
Low IL-6	16	15	13	11	6	5
High IL-6	16	13	7	5	2	0

Figure 1 High baseline interleukin (IL)-6 concentration predicts poor overall survival (OS) (3.05 versus 7.0 months, $P = 0.009$)



Numbers at risk

OS (months)	0	2	4	6	8	10
Low IL-8	16	15	13	11	6	5
High IL-8	16	13	7	5	2	0

Figure 2 High baseline interleukin (IL)-8 concentration predicts poor overall survival (OS) (3.05 versus 7.3 months, $P = 0.020$)

Results

Thirty-two patients were assessable for baseline cytokine concentrations having completed at least one treatment, with 25 assessable for cytokine response having completed at least 3 treatments (Table 1).

High expressors of IL-6 and IL-8 had significantly shorter median OS than low expressors (IL-6: 3.05 versus 7.0 months, $P = 0.009$, IL-8: 3.05 versus 7.3 months, $P = 0.02$; Figs 1 and 2). High expressors of IL-8 had significantly shorter PFS than low expressors (2.8 versus 5.6 months, $P = 0.002$). High expressors of IL-6 had a tendency to shorter PFS than low expressors (2.8 versus 5.3 months, $P = 0.06$). No other factors had any significant differences in survival between low or high expressors at baseline.

There was a significant reduction in PDGF ($P = 0.05$) and FGF ($P = 0.03$) concentrations with treatment over time using the statistical model (Figs 3 and 4). When analysed on a per-cycle basis rather than across all cycles of treatment as a whole, a PDGF concentration reduction was highly significant for each cycle. Patients who were responders for PDGF had a tendency to improved OS compared with non-responders (7.0 versus 5.4 months: log-rank $P = 0.07$; Fig. 5). Patients who were responders for FGF had a significantly improved PFS compared with non-responders (5.25 versus 1.3 months: log-rank $P < 0.001$; Fig. 6).

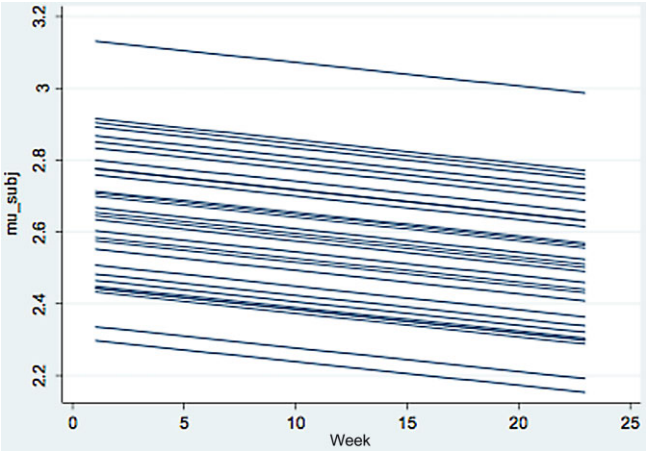


Figure 3 Reduction in serum platelet-derived growth factor (PDGF) concentration with time using a statistical model output ($P = 0.05$)

Discussion

While other studies have evaluated baseline CAF in patients with APC and correlated concentrations with clinical outcomes, this study is the first to the authors' knowledge to evaluate changes in CAF with treatment over time. In addition, this is the first study to evaluate the use of an i.v. n-3FA rich lipid emulsion in combination with gemcitabine in APC patients. Regardless of the treatment course, IL-6 and IL-8 seemed to be predictive biomarkers for clinical outcome in this cohort. Both cytokines are highly associated with inflammation, and this could represent a highly activated inflammatory state in these patients which could be a surrogate marker for infection or highly active neoplastic proliferation. Nevertheless, if this is born out in larger scale clinical

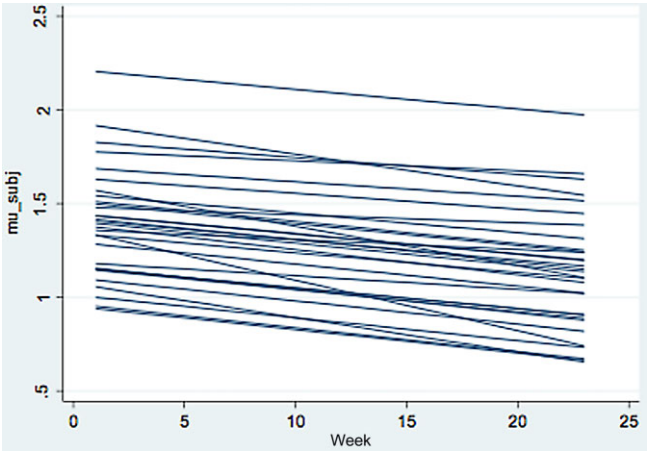
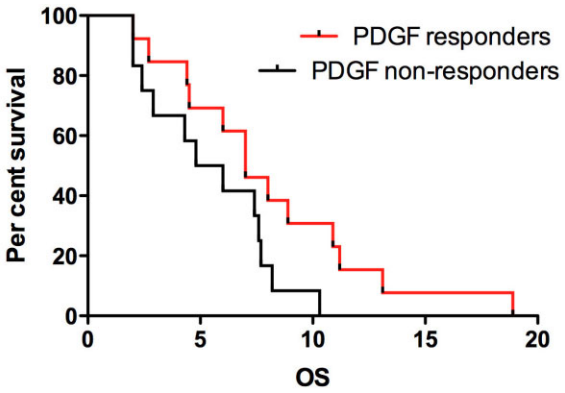


Figure 4 Reduction in serum fibroblast growth factor (FGF) concentration with time using the statistical model output ($P = 0.03$)

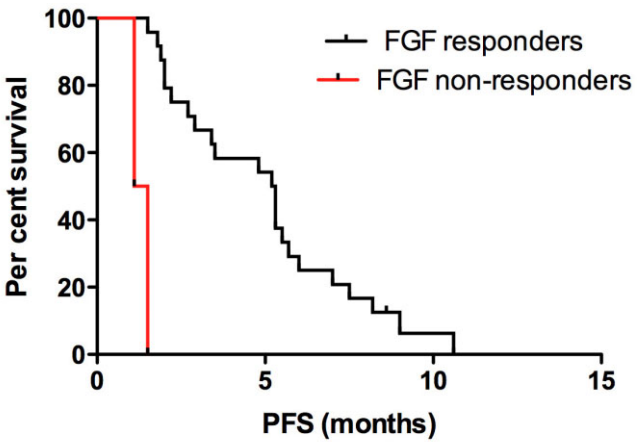


Numbers at risk

OS (months)	0	2	4	6	8	10
Responders	13	14	12	9	6	5
Non-responders	11	12	9	6	3	1

Figure 5 Overall survival (OS) of platelet-derived growth factor (PDGF) responders (7.0 months) versus non responders (5.4 months) $P = 0.070$

trials, then these CAF could provide a marker of non-response to treatment which could influence whether or not to begin treatment on those patients in whom the potential benefit was deemed be marginal. In addition, the reduction in PDGF in particular is interesting, particularly when viewed in the context of the loss of benefit during the rest week. The fact that differences in survival between PDGF responders and non-responders did not quite reach significance may be because the small numbers of patients involved.



Numbers at risk

PFS (months)	0	2	4	6	8	10
Responders	23	20	15	7	5	1
Non-responders	2	0				

Figure 6 Progression-free survival (PFS) of fibroblast growth factor (FGF) responders (5.3 months) versus non- responders (1.3 months) $P < 0.001$

Table 1 Baseline characteristics of trial patients

Male : female	19:13
Median age (range)	66 years (40–73)
Performance status 0:1	14:18
Locally advanced : metastatic disease	8:24

Limitations

There are some obvious limitations to this study. It is not clear whether the effects observed are truly a result of the treatment or if they are surrogate markers for the cohort of patients who would have had a favourable outcome irrespective of any manipulation. It is possible that the PDGF response is a marker of treatment response and therefore clinical benefit or is independent of treatment. In addition, this was a small non-randomized single arm trial, so it is impossible to ascertain the independent effect of n-3FA when added to gemcitabine and whether some property of the combination is responsible for the effect. A large-scale randomized controlled trial is planned to assess the independent effects, with both clinical and translational science outcome measures.

Conflicts of interest

All authors received industry support form BBraun, Melsungen for investigational products used in clinical trials.

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